Int J Clin Exp Med 2017;10(8):12005-12011
www.ijcem.com /ISSN:1940-5901/IJCEM0057822

Original Article

Therapeutic effects of moxibustion precondition on cerebral ischemia-reperfusion injury in rats

Xiaohong Pan[1], Chen Li[2], Feng Liu[3]

Departments of [1]Acupuncture & Moxibustion, [2]Gastroenterology, [3]Massage, Xuzhou City Hospital of TCM Affiliated to Nanjing University of Chinese Medicine, Xuzhou, Jiangsu Province, China

Received May 19, 2017; Accepted June 26, 2017; Epub August 15, 2017; Published August 30, 2017

Abstract: Objective: To study the therapeutic effects of moxibustion preconditioning on cerebral ischemia-reperfusion injury in rats. Methods: One hundred and thirty healthy male Wistar rats were selected and randomly divided into five groups: electroacupuncture preconditioning group (n=30) and moxibustion preconditioning group (n=30), aspirin preconditioning group (n=30), model group (n=30) and blank control group (n=10). Statistical analysis was performed on the neurobehavioral scores, brain water content, relative expression of miRNA664 and MMP9 in five groups. Results: Compared with the control group, the neurobehavioral scores, brain water content and relative expression of miRNA664 in electroacupuncture, aspirin preconditioning group and model group increased ($P<0.05$). Compared with the model group, the neurobehavioral scores, brain water content and relative expression of miRNA664 decreased in the electroacupuncture, aspirin preconditioning group ($P<0.05$). Compared with the control group, the expression of MMP9 in the electroacupuncture, aspirin and moxibustion preconditioning group and model group increased ($P<0.05$). Compared with the model group, the expression of MMP9 decreased in the electroacupuncture, aspirin preconditioning group ($P<0.05$). Conclusion: Moxibustion preconditioning can reduce cerebral ischemia reperfusion injury in rats, and decrease the expression of miRNA664 and MMP9.

Keywords: Moxibustion preconditioning, cerebral ischemia-reperfusion model, therapeutic effect

Introduction

More and more attention has been paid to the prophylactic treatment of ischemic cerebrovascular disease in early onset stage [1]. Acupuncture preconditioning has been widely used in clinic because of its fewer side effects on the body and easy operation. Related medical scholars set the rats as the subject. They found that acupuncture preconditioning could effectively protect nerve cells in cerebral cortex and CA region of hippocampus in rats with global cerebral ischemia; however, the specific mechanism of acupuncture's protective effects on brain remains unclear [2].

MiRNA can recognize target mRNA molecules, induce the target mRNA to inhibit translation and degrade and regulate gene expression. Ischemic preconditioning has definite brain protective effects. Studies have found that pretreatment can regulate the expression of miRNA in the brain cortex of mice [3-5]. It can down regulate the expression of target gene methyl-CpG binding protein 2 and mediate ischemic tolerance. It was reported that MeCP2 was an essential in ischemic preconditioning [6]. Related miRNA degradation could upregulate MeCP2 and participate in brain protection. However, it is not clear whether acupuncture can cause changes in miRNA expression now.

MMP9, also called type IV collagenase, can degrade ECM and participate in the formation of brain edema and destruction of blood-brain barriers. At the same time, it can destroy the microenvironment of the interaction between matrix and cells, induce the nerve cells to apoptosis, and eventually lead to neurological dysfunction. It plays an important pathophysiological role in ischemia-reperfusion injury. MiRNA-664 is an important miRNA that regulates the expression of MMP [7]. There is no report on the MMP9 formed by acupuncture pretreatment and its regulatory miRNA, miRNA664. This study is to research the corresponding

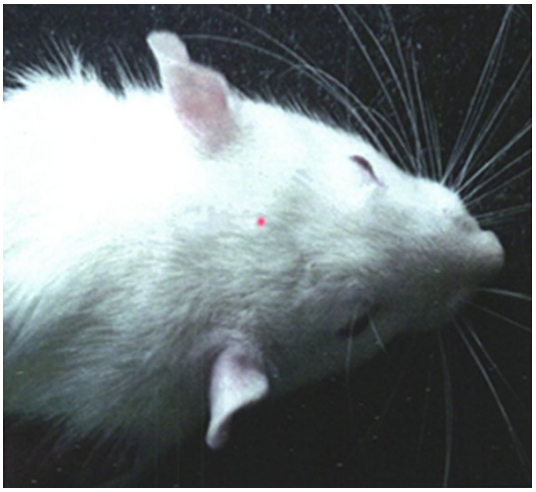

Figure 1. The electroacupuncture point of acupoint of the rat.

molecular mechanisms by observing the protective effects of acupuncture preconditioning on cerebral ischemia-reperfusion injury in rats.

Materials and methods

Animals and groups

One hundred and thirty healthy male Clean Animal Wistar rats (Shanghai Sippr-BK Laboratory Animal Co. Ltd.) were selected as the experimental subject. They aged from three to four months, with a mean age of (3.5±0.5) months. They weighted from 250 g to 300 g, with an average weight of (275±20) g. After one-week adaptive breeding, the experiment was carried out. Rats were randomly divided into five groups: electroacupuncture preconditioning group (n=30) and moxibustion preconditioning group (n=30), aspirin preconditioning group (n=30), model group (n=30) and blank control group (n=10).

Methods

Model building: The models were prepared according to the Zea Longa method [8]. The rats were divided into medication administration group and sham-operated group. They were injected with 10% chloral hydrate (3 ml/kg) in their peritoneal cavity and performed the operation after being fixed in the overhead position. Proximal end of right carotidartery, external carotid artery and its branches were separated and ligated, after median incision of neck. Right internal carotid artery (ICA) was separated, pterygopalatine artery was separated down with the ICA and its branches were ligated at the end. The nylon suture was prepared at the proximal end of ICA, and artery occlusion was put at the distal end. After the incision of carotidartery, the 4-0 nylon suture was sent to for 17-20 mm from the carotidartery into anterior cerebral artery to block all the bloodstream from the cerebral arteries. Artery occlusion was removed, nylon suture was tightened without 1 cm long thread end and then the skin was sutured. One hour after ischemia, medication administration group was injected normal saline through vein. Reperfusion one hour after ischemia, the reserved end of the suture was gently pulled to the resistance which meant it reached the carotid artery incisions, and the blood stream was opened. There was unnecessary for the secondary anesthesia and incision. There was the same procedure for the sham-operated group except sending the nylon suture. Medication administration group was administered to the stomach when ischemia. Twenty-four after reperfusion, the behavior of rats was observed and graded. Refer to Zea Longa's five-point scale: 0 for normal and without nerve injury symptom; 1 point for unable to fully extend the front paws; 2 points for turning around externally; 3 points for tilting to the opposite side; 4 points for unable to walk spontaneously and without consciousness. The standard to evaluate whether the rat model was successful or not was that the forepaws of rats could not fully stretch after the reperfusion, or rats could not stand steadily and tilt to the opposite side, or turned to the right when they walked and survived until the prescribed time.

Electroacupuncture preconditioning method: Then the Tianmen acupoint and Baihui acupoint of rats in electroacupuncture pretreatment group were performed horizontal insertions of needle for 3 mm, and Dazhui acupoint was performed an oblique insertion of needle for 3 mm. After the acupuncture, electric acupuncture therapy apparatus was connected with Baihui acupoint and Dazhui acupoint. The dilatational wave frequency and current intensity were set at 2/5 Hz and 1-2 mA respectively. Twenty minutes at every turn, once a day, one week for one course. The electroacupuncture point is shown in **Figure 1**.

Acupuncture preconditioning method: The heads of rats in moxibustion preconditioning group were fixed and the rats were kept in a

waking state. Then Tianmen acupoint, Baihui-acupoint and Dazhui acupoint were selected to be marked after they were barbered and then began the twenty-minute suspended moxibustion was carried out. In this period, Folium artemisia argyi was fully used, once a day, one week for a course.

Aspirin preconditioning method: The intragastric administration dose for rats were determined, this process was strictly in line with equivalent dose rate table (conversion of body surface area of human and animal). The aspirin dose was 0.01 g/kg. Aspirin tablets were ground to powder and mixed with distilled water. The solution concentration was 0.01 g/ml. In the course of administration, drug was strictly based on the dose required for the rats, once a day, and 1 week for a course. This group was regarded as positive control.

Blank control group method: The rats in this group were in the original state.

Observation indexes

One day after reperfusion, the neurological functions of the five groups were scored (except 10 rats in blank control group, there were 30 rats in each group). Normal gait, ataxia, crawl, no gait was recorded as 0, 1 point, 2 points, and 3 points. Spontaneous inquiry, able to move when being stimulated were recorded as 0, 1 point respectively; able to drink water, unable to drink water were recorded as 0, 1 point respectively; able to take food, unable to take food were recorded as 0, 1 point respectively; the rats can move when received pain stimulation, only trunk or head can move, no response in limb or retraction were recorded as 0, 1 point, 2 points respectively [9]. At the same time, cerebral hemisphere water content of the damaged side was calculated one day after reperfusion. In this process, firstly, the wet and dry weight method was fully utilized. To weigh out wet weight by the electronic balance. Then the specimen was placed in infrared drying oven at 95-100°C for one to two days' drying to constant weight, and the dry weight was weighed out. Brain water content was (wet weight-dry weight)/wet weight * 100% [10].

In addition, one day after reperfusion, the relative expression of miRNA664 was detected by reverse transcription real-time quantitative PCR. Firstly, one step miRNA quantitative system was established. Total RNA0.1-100 ng was performed for reverse transcription reaction. Reaction system: total RNA0.1-100 ng, 2 × Master mix 12.5 μL, 50 × SYBR GreenI 0.5 μL, RT Primer (10 μM) 0.5 μL, Forward Primer (10 μM) 0.5 μL, Reverse Primer (10 μM) 0.5 μL, DEPC-H2O was supplemented to 25 μL. Secondly, real time quantitative PCR reaction procedure was carried out. Reverse transcription for 60 minutes at 37-42°C, heat inactivation and denaturation for 10 minutes at 95°C, amplification for 20 seconds at 95°C, 30 seconds at 62°C and 30 seconds at 72°C, with totally 45 cycles. In the above steps, the reaction system and reagent dosage were strictly adopted according to the instructions of EzOmicsTM miRNA qPCR Detection Kit (Biomics Company). Primer sequence of miR664 was ATCGTACGTGGGACTGGCTAGGGAAAATGA (positive sequence) and GCAGGGTCCGAGGTATTC (negative sequence). The relative expression of miR664 was analyzed by relative quantification study. Analysis index was 2-ΔΔCt.

The expression of matrix metalloproteinase 9 (MMP9) (Cell Signaling Technology, 1:1000) in brain tissues was detected by Western Blot. We clipped 100 mg tissue, added 1 mL RIPA cell lysate (containing 1 mM PMSF) and then extracted the total protein. After SDS-PAGE gel electrophoresis and electricity trarsmembrane, the protein was transferred onto nitrocellulose membrane. We used 5% skimmed milk to seal off. After two hours, TBS was used to wash the membrane. Primary antibody MMP9 (92 kDa) and internal control beta-actin antibody were added in and saved at 4°C for one night. Scrubbing solution (TBST) was added to wash for 10 minutes each time, totally for 3 times. At last, the secondary antibody labeled by horseradish peroxidase was added and reacted for two hours. After washing the membrane, ECL reagent was added to develop.

Statistical analysis

Software SPSS20.0 was used to analyze data. Neurobehavioral scores, brain water content, relative expression of miRNA664 and MMP9 and other measurement data in five groups were expressed as standard deviation ($\bar{x} \pm s$). One-Way ANOVA combined with Bonferroni correction was applied. Inspection level was a= 0.05.

Table 1. Comparison of neurobehavioral scores among the five groups of rats ($\bar{x}\pm s$)

Group	Cases	Neurobehavioral score (point)
Electroacupuncture preconditioning group	30	1.50±0.50[a,b,c,d]
Moxibustion preconditioning group	30	1.64±0.47[b,c,d]
Aspirin preconditioning group	30	1.84±0.58[a,c,d]
Model group	30	2.50±0.50[a,b,d]
Blank control group	10	0[a,b,c]

Note: Compared with moxibustion preconditioning group, [a]P<0.05; compared with aspirin preconditioning group, [b]P<0.05; compared with model group, [c]P<0.05; compared with blank control group, [d]P<0.05.

Table 2. Comparison of brain water content among five groups of rats ($\bar{x}\pm s$)

Group	Cases	Brain water content (%)
Electroacupuncture preconditioning group	30	78.62±0.40[a,b,c,d]
Moxibustion preconditioning group	30	79.13±0.44[b,c,d]
Aspirin preconditioning group	30	79.87±0.30[a,c,d]
Model group	30	80.90±0.41[a,b,d]
Blank control group	10	77.11±0.24[a,b,c]

Note: Compared with moxibustion preconditioning group, [a]P<0.05; compared with aspirin preconditioning group, [b]P<0.05; compared with model group, [c]P<0.05; compared with blank control group, [d]P<0.05.

Results

Comparison of neurobehavioral scores among five groups of rats

The neurobehavioral scores in electroacupuncture preconditioning group, moxibustion preconditioning group, aspirin preconditioning group and model group gradually improved (P<0.05), and all were significantly higher than those in blank control group (P<0.05). Compared with electroacupuncture preconditioning group, the neurobehavioral scores in moxibustion preconditioning group and aspirin preconditioning group increased; compared with moxibustion preconditioning group, the neurobehavioral scores in aspirin preconditioning group increased. All the differences were statistical significant (P<0.05). All the details are shown in **Table 1**.

Comparison of brain water content among five groups of rats

The brain water content in electroacupuncture preconditioning group, moxibustion preconditioning group, aspirin preconditioning group and model group gradually improved (P<0.05), and all were significantly higher than those in blank control group (P<0.05). Compared with electroacupuncture preconditioning group, the brain water content in moxibustion preconditioning group and aspirin preconditioning group obviously increased; compared with moxibustion preconditioning group, the brain water content in aspirin preconditioning group increased. There were statistical significance in all the differences (P<0.05). All the details are shown in **Table 2**.

Comparison of the relative expression amount of miRNA664 among five groups of rats

The relative expression amount of miRNA664 in electroacupuncture preconditioning group, moxibustion preconditioning group, aspirin preconditioning group and model group gradually increased (P<0.05), and all were remarkably higher than that in blank control group (P<0.05). Compared with electroacupuncture preconditioning group, the relative expression amount in moxibustion preconditioning group and aspirin preconditioning group increased; compared with moxibustion preconditioning group, the relative expression amount in aspirin preconditioning group also increased. All the differences reached statistical significance (P<0.05). See **Figure 2**.

Comparison of the relative expression amount of MMP9 among five groups of rats

The relative expression amount of MMP9 in electroacupuncture preconditioning group, moxibustion preconditioning group, aspirin preconditioning group and model group gradually increased (P<0.05), and all were remarkably higher than that in blank control group (P<0.05). Compared with electroacupuncture preconditioning group, the relative expression amount of MMP9 in moxibustion preconditioning group and aspirin preconditioning group increased; compared with moxibustion preconditioning group, the relative expression amount of MMP9 in aspirin preconditioning group also incr-

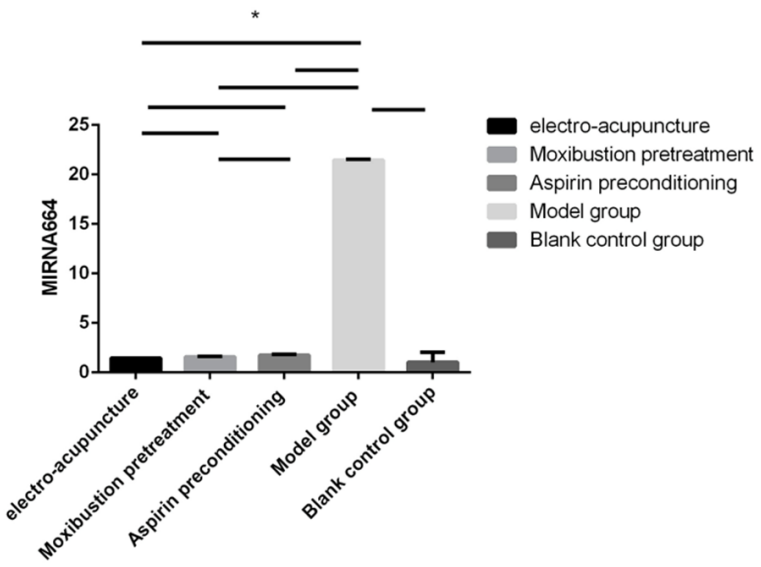

Figure 2. Comparison of the relative expression amount of miRNA664 among five groups of rats. The expression of miRNA664 of electroacupuncture preconditioning group, moxibustion preconditioning group, aspirin preconditioning group and model group were gradually improved (*P<0.05). The miRNA664 relative expression amount of these groups were significantly higher than those in blank control group (*P<0.05).

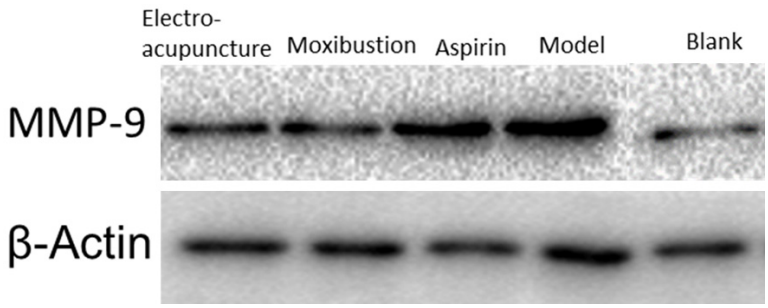

Figure 3. Comparison of the expression amount of MMP9 among five groups of rats.

eased. All the differences reached statistical significance (P<0.05). See **Figures 3**, **4**.

Discussion

MiRNA664 is induced to express during transient middle cerebral artery occlusion (MCAO) in rats and acts on Bcl-2. Related medical study showed that the translation of bcl-2/-w would be inhibited by miRNA664 [11]. The application of antagomiRNA664 in the body will elevate the effective reduction of miRNA664 levels and promote the bcl-2/-w protein levels. Thereby it provides effective preconditions for neuronal damage and infarction caused by MCAO and lightens them. In the cerebral ischemia model, miR664 was an anti-apoptotic factor and had a strong effect. In the rat models of cerebral ischemia, the miR664 levels in neurons in the ischemic marginal zone were significantly higher than those in the contralateral hemisphere. Over-expression of miR664 in cultured cortical neurons would inhibit apoptosis which was induced by OGD to a great extent, indicating that ischemic nerve cells would be protected by the over-expression of miR664.

The results of this study showed that, the neurobehavioral scores of electroacupuncture preconditioning group, moxibustion preconditioning group, aspirin preconditioning group and model group gradually improved (P<0.05), and all were significantly higher than those in blank control group (P<0.05). The brain water content of electroacupuncture preconditioning group, moxibustion preconditioning group, aspirin preconditioning group and model group gradually improved (P<0.05), and all were obviously higher than that in blank control group (P<0.05). The expression amount of miRNA664 in electroacupuncture preconditioning group, moxibustion preconditioning group, aspirin preconditioning group and model group gradually increased (P<0.05), and all were remarkably higher than those in blank control group (P<0.05). It was consistent with the relevant medical research results mentioned above.

Related medical scholars have found that the expression of MMP9 gene is regulated by miR-664, miR-125a, etc. and increased along with the increasing expression of miR-664, decreased along with the reducing expression of miR-664 by analyzing DNA chip; the consistent change between the expression of MMP9 gene and that of miR-664. It illustrated that there

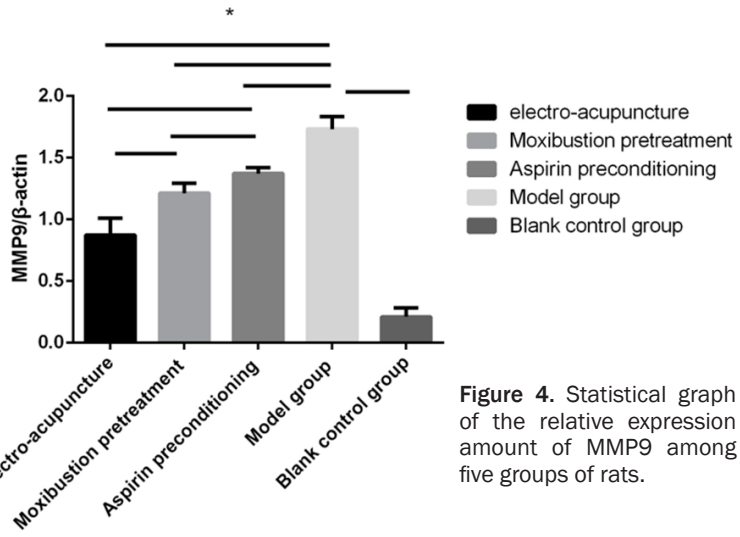

Figure 4. Statistical graph of the relative expression amount of MMP9 among five groups of rats.

might exist regulate and be regulated relationship [12-19]. Vasogenic cerebral edema and destruction of blood-brain barrier had direct and profound effects on MMP9. Accordingly, it was inferred that the possible way MiRNA involved in the formation of cerebral edema after cerebral infarction was to regulate the expression of its target gene. Gao et al. found that during cerebral ischemia reperfusion injury, the expression of MMP9 obviously increased, but decreased after the electroacupuncture [20]. The results of this study demonstrated that the expression amount of MMP9 in electroacupuncture preconditioning group, moxibustion preconditioning group, aspirin preconditioning group and model group gradually increased, and all were remarkably higher than that in blank control group; compared with electroacupuncture preconditioning group, the expression amount of MMP9 in moxibustion preconditioning group and aspirin preconditioning group increased; compared with moxibustion preconditioning group, the expression amount of MMP9 in aspirin preconditioning group also increased. These illustrated that acupuncture pretreatment could promote the neurobehavioral scores of cerebral ischemia-reperfusion rats and reduce their brain water content effectively. At the same time, it could promote the decrease of the relative expression of MMP9 by regulating the expression of miRNA664 and induce the cerebral ischemia tolerance, providing effective preconditions for the reduction of cerebral edema.

This study only observed the effects of moxibustion on the expression of MMP9 and miR-664. We did not interfere with the above-mentioned proteins and miRNA. So we couldn't get the conclusion that moxibustion mediated brain protection by specifically regulating the expression of miR-664 and MMP9. Further gene over-expression or gene knockout will contribute to clarify the possible causal links. At the same time, because of the small sample size, the results may not have universal representative significance. So it is necessary for relevant medical scholars to increase the sample size for further study.

In conclusion, moxibustion preconditioning plays an active role in the treatment of cerebral ischemia-reperfusion in rats. It is worth spreading in the clinic.

Disclosure of conflict of interest

None.

Address correspondence to: Feng Liu, Department of Massage, Xuzhou City Hospital of TCM Affiliated to Nanjing University of Chinese Medicine, No.169 Zhongshan South Road, Xuzhou 221003, Jiangsu Province, China. Tel: +86-15805200321; E-mail: liufeng6634@163.com

References

[1] Wadas TM. Emerging inflammatory biomarkers with acute stroke. Crit Care Nurs Clin North Am 2009; 21: 493-505.

[2] Dirnagl U, Becker K and Meisel A. Preconditioning and tolerance against cerebral ischaemia: from experimental strategies to clinical use. Lancet Neurol 2009; 8: 398-412.

[3] Svedin P, Hagberg H, Savman K, Zhu C and Mallard C. Matrix metalloproteinase-9 gene knock-out protects the immature brain after cerebral hypoxia-ischemia. J Neurosci 2007; 27: 1511-1518.

[4] Tan KS, Armugam A, Sepramaniam S, Lim KY, Setyowati KD, Wang CW and Jeyaseelan K. Expression profile of MicroRNAs in young stroke patients. PLoS One 2009; 4: e7689.

[5] Park KP, Rosell A, Foerch C, Xing C, Kim WJ, Lee S, Opdenakker G, Furie KL and Lo EH. Plasma and brain matrix metalloproteinase-9 after acute focal cerebral ischemia in rats. Stroke 2009; 40: 2836-2842.

[6] Jeyaseelan K, Lim KY and Armugam A. MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion. Stroke 2008; 39: 959-966.
[7] Ramos-Fernandez M, Bellolio MF and Stead LG. Matrix metalloproteinase-9 as a marker for acute ischemic stroke: a systematic review. J Stroke Cerebrovasc Dis 2011; 20: 47-54.
[8] Dharap A, Bowen K, Place R, Li LC and Vemuganti R. Transient focal ischemia induces extensive temporal changes in rat cerebral microRNAome. J Cereb Blood Flow Metab 2009; 29: 675-687.
[9] Zhang X, Christenson LK and Nothnick WB. Regulation of MMP-9 expression and activity in the mouse uterus by estrogen. Mol Reprod Dev 2007; 74: 321-331.
[10] Liu DZ, Tian Y, Ander BP, Xu H, Stamova BS, Zhan X, Turner RJ, Jickling G and Sharp FR. Brain and blood microRNA expression profiling of ischemic stroke, intracerebral hemorrhage, and kainate seizures. J Cereb Blood Flow Metab 2010; 30: 92-101.
[11] del Zoppo GJ, Milner R, Mabuchi T, Hung S, Wang X, Berg GI and Koziol JA. Microglial activation and matrix protease generation during focal cerebral ischemia. Stroke 2007; 38: 646-651.
[12] Buller B, Liu X, Wang X, Zhang RL, Zhang L, Hozeska-Solgot A, Chopp M and Zhang ZG. MicroRNA-21 protects neurons from ischemic death. FEBS J 2010; 277: 4299-4307.
[13] Eizawa T, Ikeda U, Murakami Y, Matsui K, Yoshioka T, Suzuki C, Takahashi M, Muroi K, Kamisawa O, Fuse K and Shimada K. Increase in circulating endothelial progenitor cells after aortic aneurysm repair. Heart Vessels 2004; 19: 107-110.
[14] Yin KJ, Deng Z, Huang H, Hamblin M, Xie C, Zhang J and Chen YE. miR-497 regulates neuronal death in mouse brain after transient focal cerebral ischemia. Neurobiol Dis 2010; 38: 17-26.
[15] Wojakowski W and Tendera M. Mobilization of bone marrow-derived progenitor cells in acute coronary syndromes. Folia Histochem Cytobiol 2005; 43: 229-232.
[16] Yin KJ, Deng Z, Hamblin M, Xiang Y, Huang H, Zhang J, Jiang X, Wang Y and Chen YE. Peroxisome proliferator-activated receptor delta regulation of miR-15a in ischemia-induced cerebral vascular endothelial injury. J Neurosci 2010; 30: 6398-6408.
[17] Urbich C and Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. Circ Res 2004; 95: 343-353.
[18] Badorff C, Brandes RP, Popp R, Rupp S, Urbich C, Aicher A, Fleming I, Busse R, Zeiher AM and Dimmeler S. Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. Circulation 2003; 107: 1024-1032.
[19] Lusardi TA, Farr CD, Faulkner CL, Pignataro G, Yang T, Lan J, Simon RP and Saugstad JA. Ischemic preconditioning regulates expression of microRNAs and a predicted target, MeCP2, in mouse cortex. J Cereb Blood Flow Metab 2010; 30: 744-756.
[20] Gao D, Zhang X, Jiang X, Zhang W and Peng Y. Effect of resveratrol on marix metalloproteinase 9 (MMP-9) induced by cerebral ischemia-reperfusion in mice. Chinese Journal of Neurosurgical Disease Research 2007.